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(71) Applicant *(for all designated States except US)*: **INTRADIGM CORPORATION** [US/US]; 12115 Parklawn Drive, Suite K, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SCARIA, Puthupparampil, V.** [US/US]; 9602 Swallow Point Way, Montgomery Village, MD 20886 (US). **WOODLE, Martin, C.** [US/US]; 8205 Beech Tree Road, Bethesda, MD 20817 (US). **LU, Patrick, Y.** [US/US]; 17093 Briardale Road, Rockville, MD 20855 (US). **TANG, Qingquan** [US/US]; 31 Longmeadow Drive, Gaithersburg, MD 20878 (US). **XU, Jun** [CN/US]; 18120 Coachmans Road, Germantown, MD 20874 (US). **XIE, Frank, Y.** [CN/US]; 13921 Rockingham Road, Germantown, MD 20874 (US).

(74) Agents: **BOOTH, PAUL M** et al.; HELLER EHRMAN WHITE & MCAULIFFE LLP, 1666 K Street, N.W., Suite 300, Washington, DC 20006-1228 (US).

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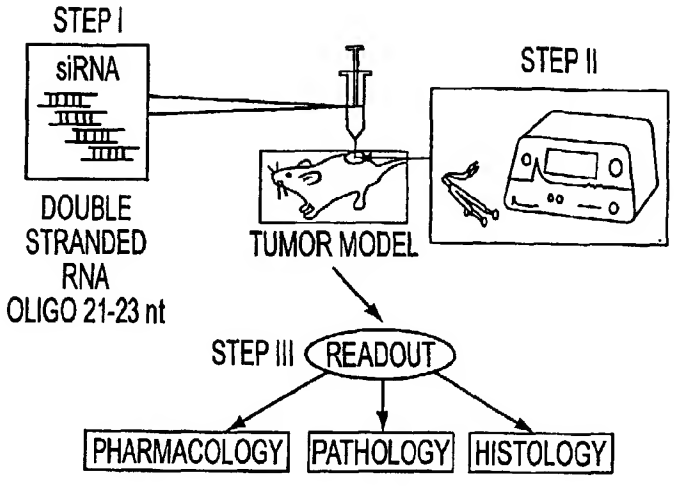
(54) Title: METHODS OF DOWN REGULATING TARGET GENE EXPRESSION IN VIVO BY INTRODUCTION OF INTERFERING RNA



(57) Abstract: Methods and compositions are provided for down regulation of target gene expression in vivo by RNA interference. The methods are useful for target discovery and validation of gene-based drug development, and for treatment of human diseases.

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Methods Of Down Regulating Target Gene Expression In Vivo By Introduction Of Interfering RNA

This application claims priority to U.S. provisional application serial number 60/401,029, filed August 06, 2002, the entirety of which is hereby incorporated by reference.

Field of the Invention

The invention provide methods and compositions for down regulating target gene expression in a subject by introducing RNA interference through *in vivo* delivery of nucleic acid, for example, by using siRNA duplexes. The methods are useful for target discovery and validation of gene-based drug development. The invention also provides methods and compositions for clinical application of siRNA therapeutics for the treatment if disease in a subject, for example to treat cancer, infectious diseases and/or inflammatory diseases.

Background of the Invention

RNA interference (RNAi) is a post-transcriptional process where a double stranded RNA inhibits gene expression in a sequence specific fashion. The RNAi process occurs in at least two steps: During the first step, a longer dsRNA is cleaved by an endogenous ribonuclease into shorter, 21- or 23-nucleotide-long dsRNAs, termed "small interfering RNAs" or siRNAs. In the second step, the smaller siRNAs then mediate the degradation of a target mRNA molecule. This RNAi effect can be achieved by introduction of either longer double-stranded RNA (dsRNA) or shorter small interfering RNA (siRNA) to the target sequence within cells. Recently, it was demonstrated that RNAi can also be achieved by introducing of plasmid that generate dsRNA complementary to target gene.

RNAi methods have been successfully used in gene function determination experiments in Drosophila [20, 22, 23, 25], C. elegans[14, 15, 16], and Zebrafish[20],. In those model organisms, it has been reported that both the chemically synthesized shorter siRNA or *in vitro* transcribed longer dsRNA can

effectively inhibit target gene expression. Methods have been reported that successfully achieved RNAi effects in non human mammalian and human cell cultures[39-56]. However, RNAi effects haveb been difficult to observe in adult animal models[57]. This is for at least two reasons: first, introduction of a long double-stranded RNA into mammalian cells triggers an antiviral response through up-regulation of interferon gene expression , resulting in apoptosis and death of the cells, and ; second, the efficiency of dsRNA delivery into the cell is too low, especially in animal disease models. Although RNAi has potential applications in both gene target validation and nucleic acid therapeutics, progress of the technology has been hindered due to the poor delivery of RNAi molecules into animal disease models. It is apparent, therefore, that improved methods for delivering RNAi molecules *in vivo* are greatly to be desired.

Summary of the Invention

It is therefore an object of the invention to provide methods for inhibiting expression of one or more specific genes in a mammal.

It is a further object of the invention to provide methods for treating disease in a mammal by inhibiting expression of one or more specific genes in the mammal.

In achieving these objects there has been provided a method for down regulating a pre-selected endogenous gene in a mammal, comprising administering to a tissue of the mammal a composition comprising a double-stranded RNA molecule where the RNA molecule specifically reduces or inhibits expression of the endogenous gene. This down regulation of an endogenous gene may be used for treating a disease in the mammal that is caused or exacerbated by expression of the gene. The mammal may be a human.

There also has been provided a method for treating a disease in a mammal associated with undesirable expression of a preselected endogenous gene, comprising applying a nucleic acid composition to a tissue of the mammal and substantially contemporaneously applying a pulsed electric field to the tissue, where the nucleic acid composition may be capable of reducing expression of the endogenous gene in the tissue. The disease may be cancer or a precancerous growth and the tissue may be, for example, a breast tissue, colon tissue, a prostate tissue, a lung tissue or an ovarian tissue.

The RNA molecule may be a small interfering RNA or a long double stranded RNA. The small interfering RNA molecule may have a length of about 21-23 bp. The long double stranded RNA may have a length of about 100 – 800 bp. The RNA may have a length of about one hundred base pairs or less.

The composition may be administered directly to a tissue of the mammal, for example via injection into a tumor or joint in the mammal.

The composition may further comprises a polymeric carrier that enhances delivery of the RNA molecule to the tissue of the mammal. The polymeric carrier may comprise a cationic polymer that binds to the RNA molecule. The cationic polymer may be an amino acid copolymer, containing, for example, histidine and lysine residues. The polymer may be a branched polymer.

The composition may contain a targeted synthetic vector that enhances delivery of the RNA molecule to the tissue of the mammal. The synthetic vector may comprise a cationic polymer, a hydrophilic polymer, and a targeting ligand. The polymer may be a polyethyleneimine, the hydrophilic polymer may be a polyethyleneglycol, and/or the targeting ligand may be a peptide comprising an RGD sequence.

In any of these methods, a pulsed electric field may be applied to the tissue substantially contemporaneously with the composition. A second electric pulse may be applied substantially contemporaneously to the tissue to enhance delivery.

The endogenous gene may be a mutated endogenous gene, and at least one mutation in the mutated gene may be in a coding or regulatory region of the gene.

The composition may be a vector composition where the vector encodes an RNA transcript operatively coupled to a regulatory sequence that controls transcription of the transcript, and where the transcript can form a double stranded RNA molecule in the tissue that specifically reduces or inhibits expression of the endogenous gene. The vector may be a viral vector or a plasmid, cosmid or bacteriophage vector. The regulatory sequence may comprise a promoter, for example a a tissue-selective promoter such as a skin-selective promoter or a tumor selective promoter. The may be selected from the group consisting of CMV, RSV LTR, MPSV LTR, SV4 AFP, ALA, OC and keratin specific promoters.

In any of these methods, the endogenous gene may be selected from the group consisting of cancer causing genes, growth factor genes, angiogenesis factor genes, protease genes, protein serine/threonine kinase genes, protein

tyrosine kinase genes, protein serine/threonine phosphatase genes, protein tyrosine phosphatase genes, receptor genes, matrix protein genes, cytokine genes, growth hormone genes, and transcription factor genes. The gene may be selected from the group consisting of VEGF, VEGF-R, VEGF-R2, VEGF121, VEGF165, VEGF189, and VEGF206.

In methods involving application of an electric pulse the pulse may comprise a square wave pulse of at least 50 V that may be applied to the tissue for between about 10 and about 20 minutes The pulse may be monopolar, bipolar or of multiple polarity. The pulse may comprise an exponential decay pulse of 120 V that may be applied to the tissue for between about 10 and about 20 minutes. In each of these methods the electric pulse may be applied via an electrode selected from the group consisting of a caliper electrode, a meander electrode, a needle electrode, a micro needle array electrode, a micropatch electrode, a ring electrode, and combinations thereof. The caliper electrode may have an area of about 1 cm^2. The caliper electrode may be applied to a skin fold having a thickness of about 1 mm to about 6 mm.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Fig. 1 shows electroporation mediated RNAi delivery in animal disease model. Step I: local delivery of naked plasmid DNA expressing double stranded RNA in host tissue with a saline solution, of double stranded RNA (large fragment-700 bp, or 21-23 nt oligos), and both; Step II: pulsed electrical field treatment with appropriate apparatus and probes; Step III: Biological readout to detect the efficiency of RNAi inhibition of targeted protein and therapeutic efficacy.

Fig. 2 shows RNAi mediated inhibition of Luciferase Expression in a Xenograft tumor model. Luciferase expression vector (pCI-Luc) was co-delivered with specific dsRNA (Luc-dsRNA) and non-specific dsRNA(LacZ-dsRNA) at 3

concentrations intra-tumor directly. At 0.5 μg, Luciferase expression was significantly inhibited by vector expressed specific dsRNA, but not by LacZ-dsRNA. When concentrations of both specific and non-specific dsRNAs reach to 5 μg dose, the inhibition become non-specific.

Fig. 3 shows down regulation of angiogenesis factor VEGF results in inhibition of tumor growth by electroporation mediated VEGF specific RNAi delivery. It becomes a very aggressive tumor line when MCF7 transduced with VEGF165 permanently. Two times electroporation with 10 μg RNAi molecules each delayed the tumor growth.

Fig. 4 shows different inhibition dynamics with siRNA or dsRNA. Although the same parameters of electroporation, the same routes of delivery and the same amount of each form of RNAi was applied, the inhibition of the tumor growth differed. DsRNA demonstrated an early strong effect verses a delayed effect mediated by siRNA. Comparing to LacZ RNAi, both dsRNA and siRNA specific to VEGF clearly demonstrated sequence specific inhibition.

Fig. 5 shows VEGFR2 specific inhibition of tumor growth. Mouse VEGFR2 gene has been considered to play a pivotal role in tumor angiogenesis and in stromal cross-talking with tumor cells. After two deliveries intratumorally of mVEGFR2 specific RNAi followed by electroporations, tumor growth clearly was delayed compared to deliveries of Luc expressing plasmid and non-specific RNAi.

Fig. 6. When siRNA duplexes (Fluorescent labeled) were delivered intratumorally with electroporation enhancement, they were evenly distributed through out the tumor. This result indicated the siRNA delivery is different from plasmid which usually only localized in a small area in the tumor.

Fig. 7 shows LacZ-specific siRNA delivery into a tumor formed by MCF-7/VEGF165 cells, which has been engineered to endogenously express LacZ. The results show that 20 μg of siRNA achieved >70 percent reduction in β-Gal expression 24 hours after delivery of LacZ-siRNA.

Fig. 8. shows immunohistochemical staining of tumor tissue treated with VEGF-siRNA and LacZ-siRNA . H&E staining demonstrated a significantly different image of VEGF-siRNA treated tumor from LacZ-siRNA treated tumor (A-B). VEGF staining (C) was lost when tumor was treated with VEGF-siRNA

(D). Apoptosis activity was significant upregulated in the VEGF-siRNA treated tumor.

Fig. 9. VEGF-siRNA knockdown VEGF expression at mRNA level *in vitro* (left panel) resulted in MDA-MB-435 breast tumor growth inhibition.

Fig. 10. Co-delivery of Luciferase expression plasmid and Luc-siRNA into the MDA-MB-435 tumor, demonstrated that Luc-siRNA achieved significant knockdown of luciferase expression.

Fig. 11. When VEGF-siRNA was delivered into a tumor model, the mRNA level of VEGF in the tumor tissue was down regulated.

Fig. 12. When ICT1031 or April gene expression was subject to RNAi knockdown, tumor growth was inhibited. A cell-based assay did not show significant change of apoptosis activity.

Fig. 13. When ICT1027 or GRB2 gene expression was subject to RNAi knockdown, tumor growth was inhibited and cell apoptosis activity was significant increased.

Fig. 14. When ICT1024 or EGF-AP gene expression was subject to RNAi knockdown, tumor growth was inhibited and cell apoptosis activity was significant increased.

Fig. 15. When ICT1030 gene expression was subject to RNAi knockdown, tumor growth was accelerated.

Fig. 16. PolyTran (HK polymer) carrier mediated ICT1003-siRNA delivery resulted in tumor inhibition compared to the GFP-siRNA treated tumor.

Fig. 17. Co-delivery of luciferase expression plasmid with luc-siRNA into mouse airway through a method called oraltracheal delivery, resulted in a siRNA-mediated luciferase inhibition in mouse lung. The luciferase activities from different samples were measured by harvesting the lungs first and then testing in a luminometer.

Fig. 18. Co-delivery of luciferase expression plasmid with luc-siRNA into mouse muscle through electroporation resulted in siRNA-mediated luciferase inhibition in mouse leg muscle.

Fig. 19. Co-delivery of luciferase expression plasmid with luc-siRNA into mouse joints through electroporation resulted in a siRNA-mediated luciferase inhibition in mouse leg joints.

Fig. 20. A systemic approach for siRNA delivery through IV injection showed tumor targeting effect. Tumor tissue is marked by two circles.

Fig. 21. Use of mouse VEGF a. mVEGFR1 and mVEGFR2 specific siRNA duplexes, through an IV systemic delivery, significantly decreased the neovasculature area of the front of eyes.

Fig. 22. Using the same method as in Fig. 21, the decrease of the red neovasculature in the RNAi- treated group clearly is greater than in the control group.

Detailed Description

Methods for efficient RNAi delivery *in vivo* are provided. In one embodiment, RNAi is delivered into a subject, for example, a human or other animal, both locally and systemically through use of a pulsed electrical field (electroporation). The methods may be used with (1) all forms of RNAi, e.g. siRNA, dsRNA and DNA-RNA duplex; (2) all forms of RNAi payloads, *e.g.* synthetic, *in vitro* transcribed and vector expressed RNAi; and (3) all types of tissues and organs that are accessible for electroporation. In other methods the RNAi is delivered using a polymer carrier and via intravenous (IV) delivery.

The invention also provides the medium used for delivery of RNAi;) routes chosen for effective delivery and parameters suitable for use for *in vivo* electroporation. The methods of the present invention have been used to achieve down regulation of a reporter gene that is co-delivered with its corresponding RNAi. The methods also have been successfully used to demonstrate antitumor efficacy after delivery of different payload forms of the corresponding RNAi by, for example, down regulation of expression of angiogenesis factors, e.g. VEGF and VEGFR2.

The present inventors have identified certain properties of different forms of RNAi, e.g. small interfering RNA (21-23 nt) and double stranded RNA (about 700 bp) in animal disease models. This invention provided a powerful tool to achieve RNAi effects *in vivo*, and hold tremendous potential for various applications in functional genomic research and in nucleic acid therapeutics.

The use of RNA interference (RNAi) has been developing rapidly in cell culture and in model organisms such as Drosophila, C. elegans, and zebrafish. Studies of RNAi have found that long dsRNA is processed by Dicer, a cellular ribonuclease III, to generate duplexes of about 21 nt with 3'-overhangs, called

short interfering RNA (siRNA), which mediates sequence-specific mRNA degradation (5). An understanding of the mechanisms of RNAi and its rapidly expanding application represent a major breakthrough during the last decade in the field of biomedicine. Use of siRNA duplexes to interfere with expression of a specific gene requires knowledge of target accessibility, effective delivery of the siRNA into the target cells and, for some biological applications, long-term activity of the siRNA in the cell.

Together with the rapidly growing literature on siRNA as a functional genomic tool, there is emerging interest in using siRNA molecules as novel therapeutics. Successful therapeutic applications will depend upon successful development of optimized local and systemic delivery methods. The advantages of using siRNA as a therapeutic agent are due to its specificity (3, 4), stability (18) and mechanism of action (5, 6).

In cancer, the tumorigenesis process is thought to be the result of abnormal over-expression of oncogenes, angiogenesis factors, growth factors, and mutant tumor suppressors, even though under-expression of other proteins also plays a critical role. Increasing evidences supports the notion that siRNA duplexes are able to "knockdown" tumorigenic genes both *in vitro* and *in vivo,* resulting in significant antitumor effects (6). The present inventors have demonstrated substantial knockdown of human VEGF in MCF-7 cell, MDA-MB-435 cell and 1483 cell induced xenograft tumor models, achieving tumor growth inhibition of 40-80%. It is anticipated that VEGF-siRNA induced antiangiogenesis effect will alter the microvasculature in tumor and will result in activation of tumor cell apoptosis and, on the other hand, will also enhance efficacy of the cytotoxic chemotherapeutic drugs. However, to achieve significantly improved antitumor efficacy of antiangiogenesis agents and chemotherapeutic drugs, a highly effective delivery method is necessary so that elevated concentrations of the drugs accumulate in the local tumor tissue.

The present inventors previously have described a method of validating drug targets that determines which targets controlling tumor disease and thus justify anti-tumor drug discovery (see PCT/US02/31554). This method validates targets directly in animal tumor models through transgene over-expression and eliminates targets lacking disease control. The method reduces the need for protein generation, antibodies, and/or transgenic animals, while providing clear

and definitive evidence that targets actually control the disease. Moreover, the method provides valuable information that may be lost with methods that rely solely on cell-culture and miss the complex interactions of multiple cell types that result in disease pathology.

The present inventors also have described gene delivery technologies suitable for high throughput delivery into animal tumor models. See WO01/47496, the contents of which are hereby incorporated by reference in their entirety. These methods enable direct tumor administration of plasmids and achieve a significant (for example, seven-fold) increase in efficiency compared to "gold standard" nucleotide delivery reagents. Accordingly, the methods provide strong tumor expression and activity of candidate target proteins in the tumor.

This platform is a powerful tool for validation of genes that are under-expressed in tumor tissue. However, a method to achieve gene silencing is highly desired for validation of genes that are over-expressed in tumor tissue. Recently, double stranded RNA has been demonstrated to induce gene-specific silencing by a phenomenon called RNA interference (RNAi). Although the mechanism of RNAi is still not completely understood, early results suggested that this RNAi effect may be achieved *in vitro* in various cell types including mammalian cells.

A double stranded RNA targeted against a target mRNA results in the degradation of the target, thereby causing the silencing of the corresponding gene. Large double stranded RNA is cleaved into smaller fragments of 21-23 nucleotides long by a RNase III like activity involving an enzyme Dicer. These shorter fragments known as siRNA (small interfering RNA) are believed to mediate the cleavage of mRNA. Although gene down regulation by RNAi mechanism has been studied in C. *elegans* and other lower organisms in recent past, its effectiveness in mammalian cells in culture has only recently been demonstrated. An RNAi effect recently was demonstrated in mouse using the firefly luciferase gene reporter system(57).

To develop an RNAi technology platform for *in vivo* gene function validation and for potential clinical application of nucleic acid therapeutics to treat human diseases , the present inventors performed several *in vivo* studies in tumor-bearing mouse models. In those experiments, either siRNA or dsRNA targeting a tumor related ligand (human VEGF) or receptor (mouse VEGFR2) was intratumorally delivered to nude mice bearing xenografted human MCF-7 derived

tumor or human MDA-MB-435 tumors. For the first time we were able to demonstrate that RNAi can effectively silencing target gene in tumor cells *in vivo* and that, as a result, tumor growth was inhibited.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

Example 1. Luciferase reporter gene silencing in xenografted tumors mediated by co-transfected dsRNA

To investigate whether interfering RNAs inhibit gene expression in a mouse tumor model, we used direct intratumoral injection followed by electroporation to co-deliver naked dsRNA and Luciferase expression plasmid DNA into human MDA-MB-435 tumor xenografted in nude mice. Briefly, a 700 bp DNA fragment derived from the firefly Luciferase gene was PCR amplified and a T7 promoter sequence was added to both ends of the DNA fragment during the PCR reaction. The DNA fragment was then used as a DNA template for *in vitro* transcription. The *in vitro* transcription was carried out using an dsRNA generation kit from New England BioLab following the manufacturer's instructions. Two μg of luciferase expression plasmid, pCILuc, was mixed with 0.5, 2, and 5 μg dsRNA derived from Luciferase gene or LacZ gene in a final volume of 30 μl physiologic saline. The DNA/dsRNA mixture in saline solution was directly injected into human MDA-MB-435 tumor xenografted in Ncr Nu/Nu mice with a precision injector (Stepper, Tridake).

Immediately after injection, a procedure of pulsed electrical field was carried out (Figure 1). A thin layer of conductive gel (KY Jelly) was applied to the tumor surface to ensure good contact between the plate electrodes and tumor, and electric pulses were delivered through two external plate electrodes placed at each sides of tumor using an electroporator (BTX ECM 830, San Diego) . The parameters for electroporation were as follows: voltage to electrode distance ratio (Electric-Field Strength) was 200-V/cm; duration of each pulse was 20ms; Interval time between two pulses was 1 second (1 Hz). The number of pulses was 6. Twenty-four hours post DNA injection, tumors were excised after the animals being sacrificed. Each tumor was homogenized in 800 μl of 1x lysis buffer (Promega) in a homogenizing tube (Lysing Matrix D, Q-BIOgene) using a Fastprep (Q-BIOgene) with speed at 4 for 40 seconds at 4°C. The homogenates

were centrifuged at 14,000 rpm for 2 minutes after incubation on ice for 30 minutes. The supernatant was transferred into a fresh tube and 10 μl was used for luciferase activity assay using the Luciferase assay kit (Promega) and a Luminometer (Monolight 2010, Analytic Luminescence Lab.).

As illustrated in Figure 2, the co-delivered dsRNA derived from Luciferase gene was able to silence Luciferase expression in a xenografted tumor. As little as 0.5 μg dsRNA was enough to achieve significant gene silencing against 2 μg of co-delivered pCILuc plasmid DNA. Non-specific dsRNA interference effect was observed when 5 μg dsRNA derived from LacZ gene was co-delivered with 2 μg of pCILuc plasmid DNA. No non-specific effect was observed at lower doses of dsRNA (0.5 μg and 2 ug). This is the first observation of dsRNA mediated specific gene silencing in xenografted tumor in adult mice.

Example 2: RNAi mediated human VEGF gene silencing inhibits human MCF-7 derivate tumor growth in mice

An *in vivo* study was carried out to demonstrate that the introduced siRNA can not only silence the co-delivered reporter gene, but also down regulate expression of an endogenous gene, e.g. VEGF. When the target gene is a tumor control gene, down regulation of the gene causes a therapeutic efficacy: inhibition of tumor growth. Human VEGF induces angiogenesis and endothelial cell proliferation and plays an important role in regulating vasculogenesis. There are several splice variants of human VEGF including VEGF121, VEGF165, VEGF189, and VEGF206, each one comprising a specific exon addition. VEGF165 is the most predominant protein, though the transcript of VEGF121 may be more abundant. VEGF165 is a heparin-binding glycoprotein that is secreted as a homodimer of 45 kDa. Most types of cells, but usually not endothelial cells themselves, secrete VEGF. Since the first-discovered VEGF, VEGF165, increases vascular permeability, it is also known as vascular permeability factor. In addition, VEGF causes vasodilatation, partly through stimulation of nitric oxide synthase in endothelial cells. VEGF also can stimulate cell migration and inhibit apoptosis.

Two animal models were used for a comparative study. The first tumor model was established with a MCF-7 breast tumor line, and the second tumor model was established with a MCF-7 derived tumor cell line, MCF-7/VEGF165. Before injection of any type of RNAi, we observed a much more aggressive tumor

growth for MCF-7/VEGF165 induced tumor than that induced by MCF-7 itself. This behavior has been reported and represents the role of VEGF165 as a tumor growth enhancer through an angiogenesis promoting activity. To achieve a VEGF specific down regulation, 10 μg of either siRNA (21 nt) derived from hVEGF gene or siRNA derived from LacZ gene was directly injected into xenografted MCF-7/VEGF165 tumor that over-expressing human VEGF165 in nude mice. Two siRNA (21 nt) sequences were designed to target human VEGF165 gene. $VEGF_{RNAi}A$ sequence is 5'- ucgagacccugguggacauuu-3' and $VEGF_{RNAi}B$ sequence is 5'-ggccagcacauaggagagauu-3'. Both siRNAs were double-stranded with two UU overhang on both ends. For intratumoral injection, 5 μg of each of the two siRNAs makes up 10 μg of the VEGF specific siRNAs. In addition, the same amount of dsRNA (10 μg) targeting VEGF165 gene was also introduced by the same delivery method. Electric pulses were applied to tumor immediately after siRNA injection as described above. A second siRNA administration was performed on day 7 post first RNAi administration. The tumor volume was measured as an indication of hVEGF gene silencing.

As demonstrated in Figure 3, MCF-7/VEGF165 induced tumors treated with non-specific LacZ siRNA grew much faster than MCF-7 induced tumors. Administration of VEGF specific siRNA and dsRNA clearly demonstrated tumor growth inhibition effect. Two RNAi administrations at day 9 and day 16 achieved *in vivo* inhibition of tumor growth. Interestingly, treatments with VEGF specific siRNA and dsRNA yielded different inhibition patterns. Treatment with VEGF siRNAs demonstrated a delayed effect which shown stronger inhibition after day 23. On the other hand, treatment with VEGF dsRNA presented an earlier inhibition even after the first administration (Figure 4). This demonstrated that hVEGF siRNAs and hVEGF dsRNA specifically silenced the hVEGF gene in the treated tumors and therefore slowed tumor growth through the anti-angiogenesis mechanism.

Example 3: RNAi mediated mouse VEGFR2 gene silencing inhibits human MDA-MB-435 tumor growth in mice

To illustrate the power of RNAi mediated gene silencing in effecting tumor growth by targeting endogenous tumor control gene, one *in vivo* study was carried out to silence mouse VEGFR2 gene in MCF-7 derivative tumor bearing nude mice. Two siRNAi were designed to target mouse VEGFR2 gene.

$VEGFR2_{RNAi}$A sequence is 5'-gcucagcacacagaaagacuu-3' and_$VGFR2_{RNAi}$ B sequence is 5'-ugcggcgguggugacaguauu-3'. Both siRNAs were double-stranded with two UU overhang on both ends. Five μg of each siRNA makes up 10 μg for each delivery . Ten μg of siRNAi derived from mVEGFR2 or LacZ gene, or 10 μg of pCILuc plasmid DNA, was directly injected into . xenografted human MCF-7 derived tumor in nude mice. Electric pulses were applied to tumor immediately after siRNAs/DNA injection as described above. A second siRNAs/DNA administration was performed on day 7 post first administration. The tumor volume was measured as an indication of mVEGFR2 gene silencing. As demonstrated in Figure 5, tumors treated with siRNAs derived from mVEGFR2 gene grown significantly slower compared to tumors treated with pCILuc plasmid DNA or siRNAs derived from LacZ gene. LacZ siRNAs treatment did not inhibit tumor growth, therefore demonstrating that mVEGFR2 siRNAs specifically silenced mVEGFR2 gene in treated tumor and thus slow down tumor growth rate.

To further illustrate the power of RNAi mediated gene silencing in affecting tumor growth by a targeting tumor control gene, another *in vivo* study was carried out to silence the mouse VEGFR2 gene in human MDA-MB-435 tumor-bearing nude mice. In addition to siRNAs derived from mVEGFR2 decribed above, 10 μg of either dsRNA (700 nt in length) derived from mouse VEGFR2 gene or siRNAs derived from LacZ gene was directly injected into human MDA-MB-435 xengrafted tumor in nude mice. Electric pulses were applied to tumor immediately after dsRNA/siRNAs injection as described above. A second dsRNA/siRNAi administration was performed on day 3 post first administration. Ten μg of a DNAzyme specifically targeting mouse VEGFR2 was used as a positive control for down-regulation of the mVEGFR2 gene. The tumor volume was measured as an indication of mVEGFR2 gene silencing.

As demonstrated in Figure 6, tumors treated with dsRNA derived from mVEGFR2 gene grown significantly slower compared to tumors treated with siRNAs derived from LacZ gene. Furthermore, tumors treated with dsRNA derived from mVEGFR2 gene also grown significantly slower compared to tumors treated with mVEGFR2 DNAzyme. On the other hand, tumors treated with siRNAs derived from mVEGFR2 grown at comparable rate with tumors treated with mVEGFR2 DNAzyme, but still significantly slower than tumors treated with siRNAs derived from LacZ gene (Figure 6). Since the LacZ siRNAs

treatment did not inhibit tumor growth, it is our conclusion that both mVEGFR2 dsRNA and mVEGFR2 siRNAs specificly silence mVEGFR2 gene in treated MDA-MB-435 tumor and thus slow down tumor growth rate. More biochemistry assays are now being carried out to demonstrate that mVEGFR2 gene in tumor tissue were indeed specificly silenced by dsRNA derived from mVEGFR2 gene.

Example 4: PolyTran-mediated RNAi delivery inhibits human MDA-MB-435 tumor growth in mice

RNAi against targets can be successfully delivered using polymer-mediated delivery as shown by the results in Figure 16. RNAi directed against the target ICT1003 was delivered to tumor cells using a PolyTran reagent (histidine-lysine copolymer). Briefly, the methods and reagents described in WO01/47496 (which reference is incorporated herein in its entirety) were employed to deliver RNAi to the tumor model described above. GFP-siRNA was used as a control. As shown in Figure 16, RNAi directed against ICT1003 inhibited tumor growth compared to control. The results shown in Figure 16 were obtained using the branched reagent HK4b (described in WO01/47496) having the structure $[(HK)_4KGK(HK)_4]_4K_3$. The skilled artisan will recognize that other HK copolymers may be used and that other cationic polymers known in the art also may be used.

Example 5: Systemic delivery of RNAi using a targeted synthetic vector

Targeted synthetic vectors of the type described in WO01/49324, which is hereby incorporated by reference in its entirety, may be used for systemic delivery of RNAi. Briefly, a PEI-PEG-RGD (polyethyleneimine-polyethylene glycol-argine-glycine-aspartic acid) synthetic vector was prepared as described, for example, in Examples 53 and 56 of WO01/49324. This vector was used to deliver RNAi systemically via intravenous injection. The results are shown in Figures 20-22, which show that anti-VEGF RNAi molecules could successfully delivered using this targeted synthetic vector approach. The skilled artisan will recognize that other targeted synthetic vector molecules known in the art may be used. For example, the vector may have an inner shell made up of a core complex comprising the RNAi and at least one complex forming reagent. The vector also may contain a fusogenic moiety, which may comprise a shell that is anchored to the core complex, or may be incorporated directly into the core complex. The vector may further have an outer shell moiety that stabilizes the vector and

reduces nonspecific binding to proteins and cells. The outer shell moiety may comprise a hydrophilic polymer. and/or may be anchored to the fusogenic moiety. The outer shell moiety may be anchored to the core complex. The vector may contain a targeting moiety that enhances binding of the vector to a target tissue and cell population. Suitable targeting moieties are known in the art and are described in detail in WO01/49324.

Other methods of RNAi delivery

For certain applications, RNAi may be administered directly as a "naked" reagent with or without electroporation. This can be used, for example, to deliver RNAi molecules and vectors encoding RNAi molecules via direct injections into, for example, tumor tissue and directly into a joint. The RNAi may be in a suitable carrier such as, for example, a saline solution or a buffered saline solution.

Target Validation

The ultimate goal of drug target validation is demonstration that a candidate target actually controls the disease. Disease-controlling targets are the high value targets that justify drug discovery. The goal of drug development is products that selectively target key pathways and the key controlling elements of those pathways in order to provide effective therapeutic control of the disease. Validation of such key pathways and elements requires demonstration that addition or subtraction of individual candidate targets controls the disease, i.e. results in a clear increase or decrease of pathology. *In vitro* cell-based strategies have provided useful information in helping identify and select potential targets. However, the ability of targets to control *in vitro* cell models associated with disease frequently is not sufficient to prove the target actually controls the disease process, i.e. the complex interactions of multiple cell types that result in disease pathology. Definitive demonstration of disease control by targets can only be obtained by studies of those targets in a true disease model.

The process of target discovery has been greatly accelerated by genomic methods but validation remains a bottleneck. First-generation genomic methods have generated large pools of candidate targets piled up at the validation step. Many approaches are currently being used to study the function of these gene targets and to validate their role in a disease process. Many of these approaches, although having the benefit of being efficient and high throughput, often succeed only at establishing a correlation or association with disease processes rather than

determining a controlling role. Newer gene knockdown and forward or inverse genomic approaches have proven useful but these identify genes whose inhibition or mutation may have a disease role, missing potential valuable information from a gene's over-expression. Furthermore, they also employ primarily *in vitro* cell-based phenotypes, which do not reflect the complex multi-cellular mechanisms of most diseases, such as tumor angiogenesis, and hence run the risk of missing important targets in adjacent cellular pathways or provide disease associations which are incomplete without the full biological context.

Rapid Definitive Target Validation

The present methods can be used for validating cancer-related drug targets. The methods validate targets directly in animal tumor models by silencing endogenous gene(s) in tumor tissue, and can be used in tandem with methods that involve gene overexpression. See PCT/US02/31554 . These methods reduce the need for the costly and slow steps of definitive validation, such as gene cloning and sequencing, generation of proteins and antibodies or transgenic animals. The combination of these two methods vastly accelerates the process, and most importantly rapidly eliminates weaker targets. Moreover, results obtained by the methods provide clear and definitive evidence that targets actually control the disease, the key validation needed to proceed to the costly steps of drug discovery. The methods can be used to complete the validation of any candidate targets such as those generated from cell culture, model organisms, transgenic animals, etc.

Target Discovery: Capturing Targets Missed in Preliminary Validation

Another consideration is that, unfortunately, many high value disease-controlling targets may be lost when *in vitro* or disease-association methods are employed as the first "filter" in target discovery and validation. Many disease-controller targets may only be found in the context of the entire disease model. For example, targets controlling angiogenesis of tumors will only be found at the conjunction of tumors and blood vessels. In the case of tumors, certain valuable targets may only be discovered by studying the *in vivo* biological system containing assembly of tumor and surrounding tissues.

High throughput Target Discovery Solutions

We have also proposed solutions to the challenge of discovering disease controller targets. The solution is to scale-up the basic approach by applying it to screen larger sets of gene targets in a higher throughput operation. By scaling the

method to processing multiple candidate genes in animal tumor models, this approach can provide the opportunity to skip, in many cases, preliminary functional validation methods.

Tumor Target Elimination

The present methods, alone or in combination with the methods described in PCT/US02/31554, permit candidate targets to be rapidly tested for their capacity to control tumor growth. Those candidates showing only weak or negligible control of tumor growth can be eliminated from consideration in favor of those that have a strong effect on tumor growth. These Tumor Target Discrimination Methods rapidly discriminates targets into three categories: those enhancing tumor growth, those with little effect on tumor growth, and those inhibiting tumor growth.

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What is claimed is

1. A method for down regulating a pre-selected endogenous gene in a mammal, comprising administering to a tissue of said mammal a composition comprising a double-stranded RNA molecule wherein said RNA molecule specifically reduces or inhibits expression of said endogenous gene.

2. The method according to claim 1, wherein said RNA molecule is a small interfering RNA or a long double stranded RNA.

3. The method according to claim 2, wherein said RNA molecule is a small interfering RNA molecule having a length of about 21-23 bp.

4. The method according to claim 2, wherein said RNA molecule is a long double stranded RNA having a length of about 100 – 800 bp.

5. The method according to any preceding claim wherein said composition is administered directly to a tissue of said mammal.

6. The method according to claim 5, wherein administration is via injection into a tumor in said mammal or into a joint in said mammal.

7. The method according to any preceding claim wherein said composition further comprises a polymeric carrier that enhances delivery of said RNA molecule to said tissue of said mammal.

8. The method according to claim 7 wherein said polymeric carrier comprises a cationic polymer that binds to said RNA molecule.

9. The method according to claim 8 wherein said cationic polymer is an amino acid copolymer.

10. The method according to claim 9 wherein said polymer comprises histidine and lysine residues.

11. The method according to claim 10 wherein said polymer is a branched polymer.

12. The method according to any of claims 1-5 wherein said composition comprises a targeted synthetic vector that enhances delivery of said RNA molecule to said tissue of said mammal.

13. The method according to claim 12, wherein said vector comprises a cationic polymer, a hydrophilic polymer, and a targeting ligand.

14. The method according to claim 12, wherein said cationic polymer is a polyethyleneimine.

15. The method according to claim 12, wherein said hydrophilic polymer is a polyethyleneglycol.

16. The method according to claim 15, wherein said targeting ligand is a peptide comprising an RGD sequence.

17. The method according to any preceding claim wherein a pulsed electric field is applied to said tissue substantially contemporaneously with said composition.

18. The method according to any preceding claim wherein said endogenous gene is a mutated endogenous gene.

19. The method according to claim 18 wherein at least one mutation in said mutated gene is in a coding or regulatory region of said gene.

20. The method according to claim 17, further comprising substantially contemporaneously applying a second electric pulse to said tissue.

21. A method for down regulating a pre-selected endogenous gene in a mammal, comprising administering to a tissue of said mammal a vector composition wherein said vector encodes an RNA transcript operatively coupled to a regulatory sequence that controls transcription of said transcript,

and wherein said transcript can form a double stranded RNA molecule in said tissue that specifically reduces or inhibits expression of said endogenous gene.

22. The method according to claim 21, wherein said vector is a viral vector or a plasmid, cosmid or bacteriophage vector.

23. The method according to any preceding claim, wherein said endogenous gene is selected from the group consisting of cancer causing genes, growth factor genes, angiogenesis factor genes, protease genes, protein serine/threonine kinase genes, protein tyrosine kinase genes, protein serine/threonine phosphatase genes, protein tyrosine phosphatase genes, receptor genes, matrix protein genes, cytokine genes, growth hormone genes, and transcription factor genes.

24. The method according to claim 21, wherein said regulatory sequence comprises a promoter.

25. The method according to claim 24 wherein said promoter is a tissue-selective promoter.

26. The method according to claim 25 wherein said tissue-selective promoter is a skin-selective promoter or a tumor selective promoter.

27. The method according to claim 24, wherein said promoter is selected from the group consisting of CMV, RSV LTR, MPSV LTR, SV40, AFP, ALA, OC and keratin specific promoters.

28. The method according to claim 17, wherein said electric pulse comprises a square wave pulse of at least 50 V that is applied to said tissue for between about 10 and about 20 minutes.

29. The method according to claim 28, wherein said electric pulse is monopolar, bipolar or of multiple polarity.

30. The method according to claim 17 wherein said electric pulse comprises an exponential decay pulse of 120 V that is applied to said tissue for between about 10 and about 20 minutes.

31. The method according to claim 17, wherein said electric pulse is applied via an electrode selected from the group consisting of a caliper electrode, a meander electrode, a needle electrode, a micro needle array electrode, a micropatch electrode, a ring electrode, and combinations thereof.

32. The method according to claim 31 wherein said electrode is a caliper electrode having an area of about 1 cm^2.

33. The method of claim 32 wherein the caliper electrode is applied to a skin fold having a thickness of about 1 mm to about 6 mm.

34. A method for treating a disease in a mammal associated with undesirable expression of a preselected endogenous gene, comprising applying a nucleic acid composition to a tissue of said mammal and substantially contemporaneously applying a pulsed electric field to said tissue, wherein said nucleic acid composition is capable of reducing expression of the endogenous gene in said tissue.

35 The method according to claim 34, wherein said disease is cancer or a precancerous growth.

36. The method according to claim 34, wherein said tissue is a breast tissue, colon tissue, a prostate tissue, a lung tissue or an ovarian tissue.

37. The method according to claim 34, wherein said nucleic acid composition comprises a small interfering RNA, a long double stranded RNA, or a polynucleotide molecule that encodes an RNA transcript that can form a substantially double stranded RNA molecule.

38 The method according to claim 37, wherein said RNA molecule is a small interfering RNA molecule having a length of about 21-23 bp.

39. The method according to claim 37, wherein said RNA molecule is a long double stranded RNA having a length of about 100 – 800 bp.

40. The method according to claim 39, wherein said RNA has a length of about one hundred base pairs or less.

41. The method according to claim 34, wherein said nucleic acid composition is a vector capable of encoding an siRNA or an RNAi, and wherein said vector is a plasmid, cosmid, bacteriophage, or viral vector.

42. The method according to claim 41, wherein said vector is a retroviral or adenoviral vector.

43. The method according to any preceding claim, wherein said mammal is a human.

44. The method according to claim 34, wherein said preselected endogenous gene is selected from the group consisting of cancer causing genes, growth factor genes, angiogenesis factor genes, protease genes, protein serine/threonine kinase genes, protein tyrosine kinase genes, protein serine/threonine phosphatase genes, protein tyrosine phosphatase genes, receptor genes, matrix protein genes, cytokine genes, growth hormone genes, and transcription factor genes.

45. The method according to claim 34, wherein said gene is selected from the group consisting of VEGF, VEGF-R, VEGF-R2, VEGF121, VEGF165, VEGF189, and VEGF206.

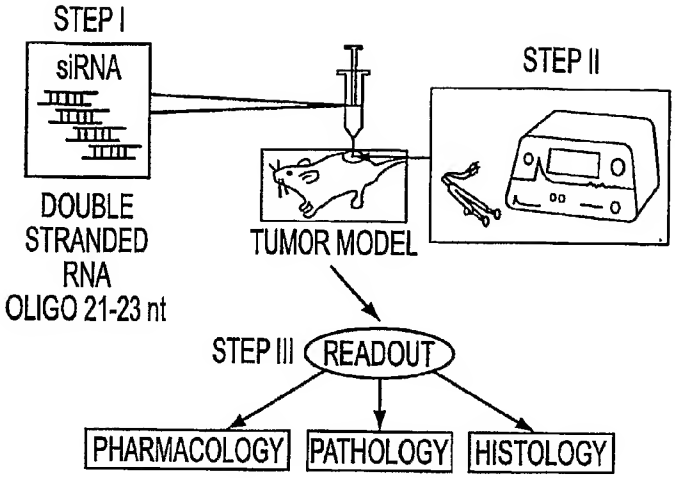

STEP I
siRNA
DOUBLE STRANDED RNA OLIGO 21-23 nt
STEP II
TUMOR MODEL
STEP III
READOUT
PHARMACOLOGY
PATHOLOGY
HISTOLOGY

FIG. 1

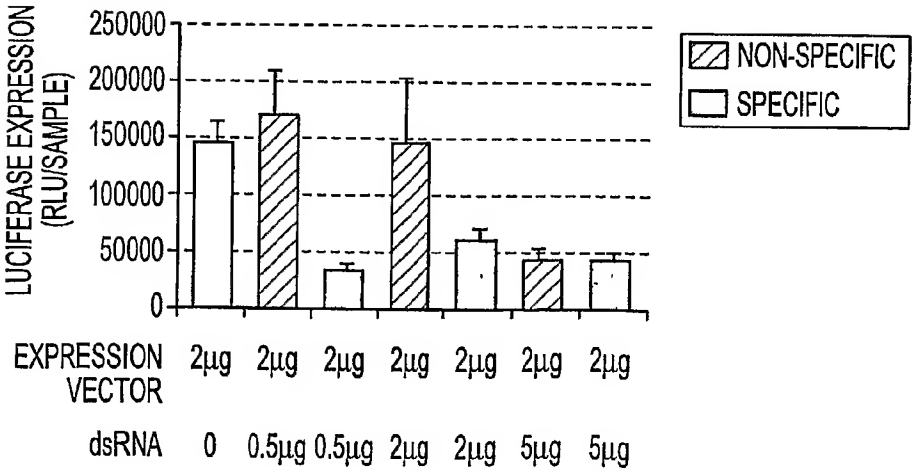

LUCIFERASE EXPRESSION (RLU/SAMPLE)
250000
200000
150000
100000
50000
0
NON-SPECIFIC
SPECIFIC
EXPRESSION VECTOR 2µg 2µg 2µg 2µg 2µg 2µg 2µg
dsRNA 0 0.5µg 0.5µg 2µg 2µg 5µg 5µg

FIG. 2

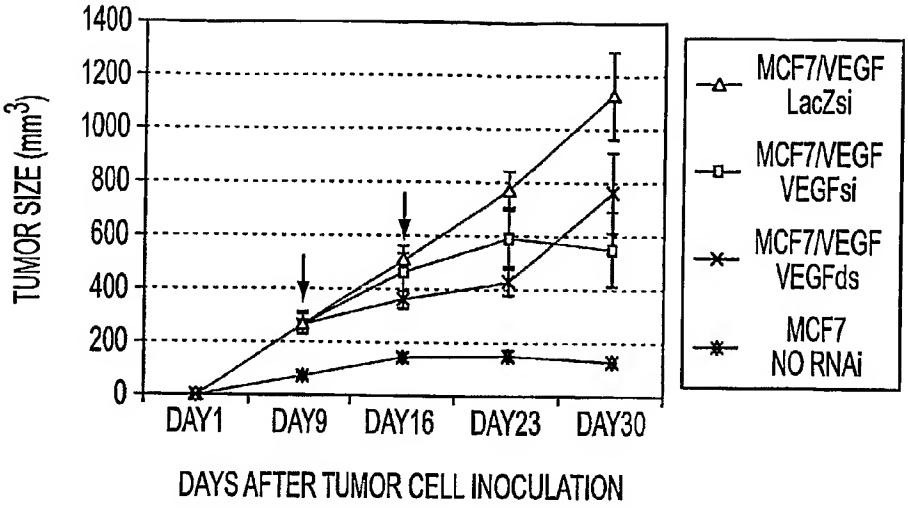

1400
1200
1000
800
600
400
200
0
TUMOR SIZE (mm3)
DAY1
DAY9
DAY16
DAY23
DAY30
DAYS AFTER TUMOR CELL INOCULATION
MCF7/VEGF LacZsi
MCF7/VEGF VEGFsi
MCF7/VEGF VEGFds
MCF7 NO RNAi

FIG. 3

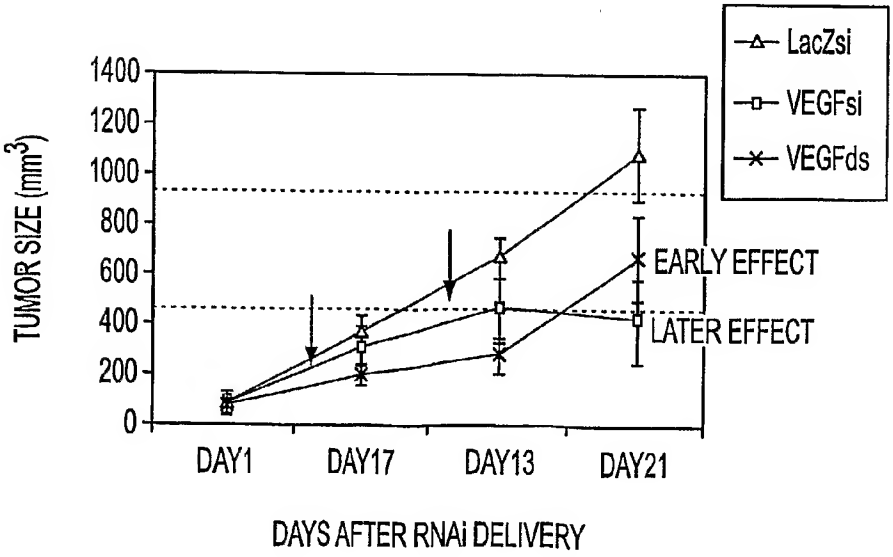

1400
1200
1000
800
600
400
200
0
TUMOR SIZE (mm3)
DAY1
DAY17
DAY13
DAY21
DAYS AFTER RNAi DELIVERY
LacZsi
VEGFsi
VEGFds
EARLY EFFECT
LATER EFFECT

FIG. 4

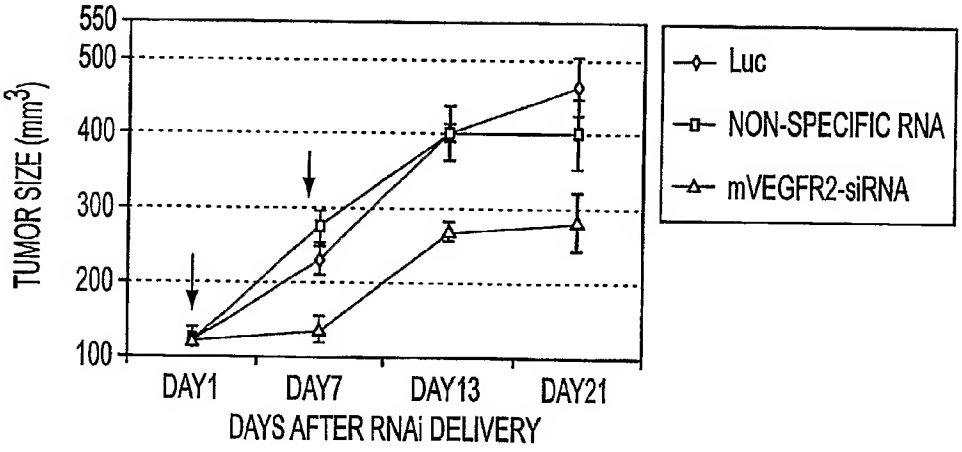
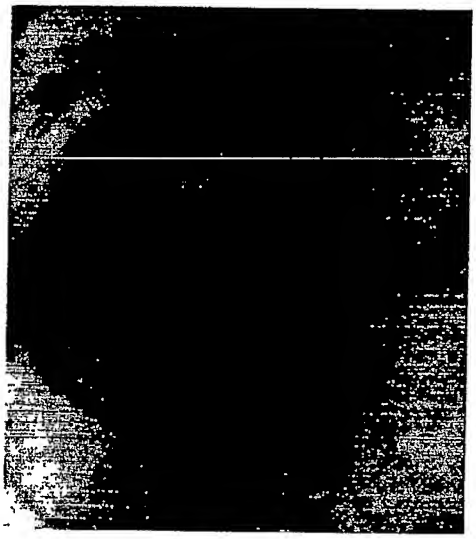
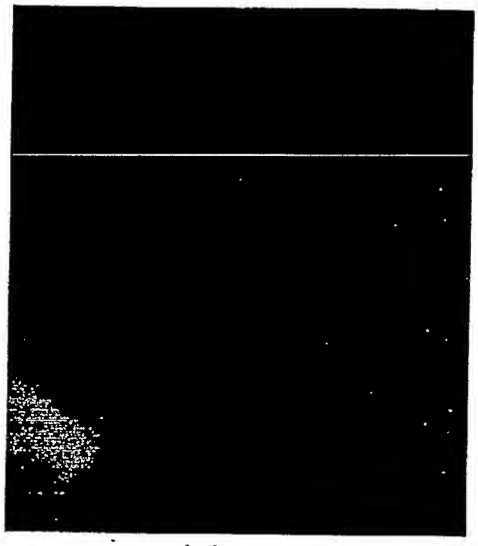

550
500
400
300
200
100
TUMOR SIZE (mm3)
DAY1
DAY7
DAY13
DAY21
DAYS AFTER RNAi DELIVERY
Luc
NON-SPECIFIC RNA
mVEGFR2-siRNA

FIG. 5



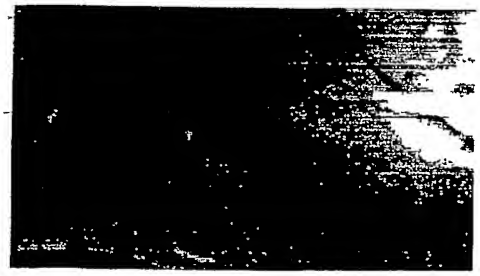
siRNA DELIVERY INTO TUMOR

FIG. 6A



siRNA DELIVERY INTO TUMOR

FIG. 6B



siRNA DELIVERY INTO TUMOR

FIG. 6C

siRNA DELIVERY INTO TUMOR

FIG. 6D

NO TREATMENT

FIG. 7A

LacZ-siRNA

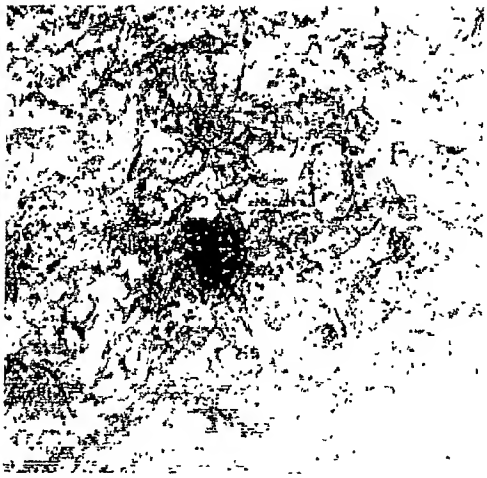


FIG. 7B

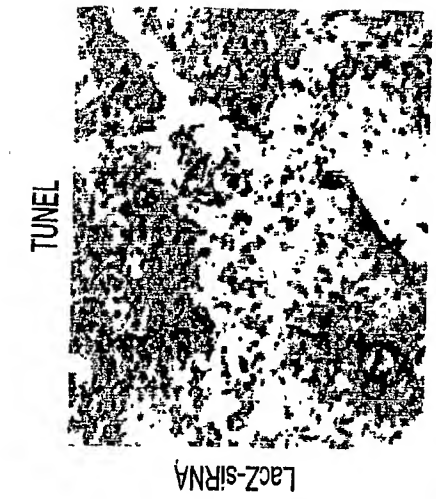


FIG. 8E

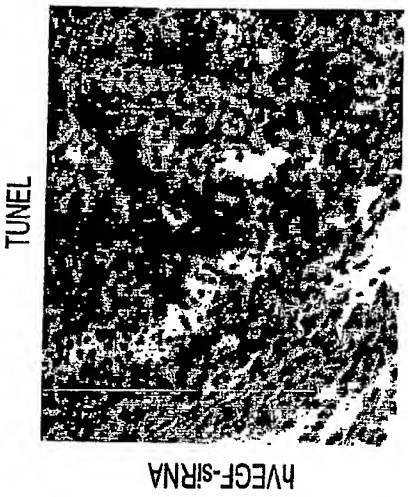


FIG. 8F

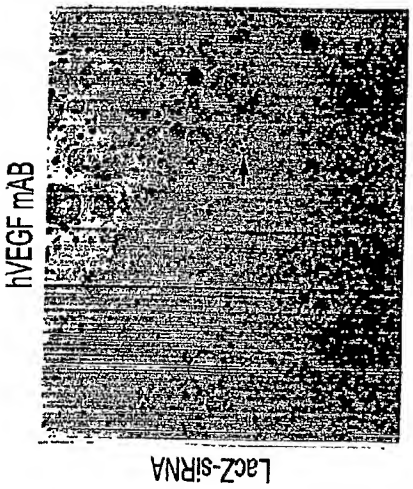


FIG. 8C

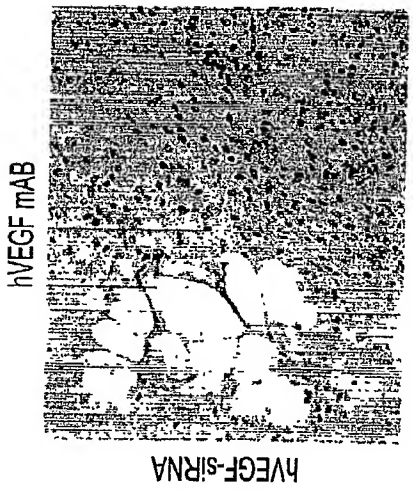


FIG. 8D

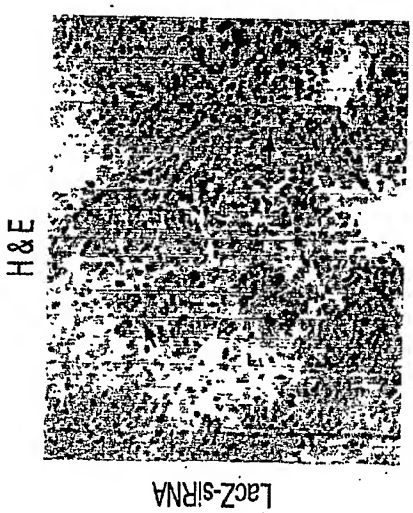


FIG. 8A

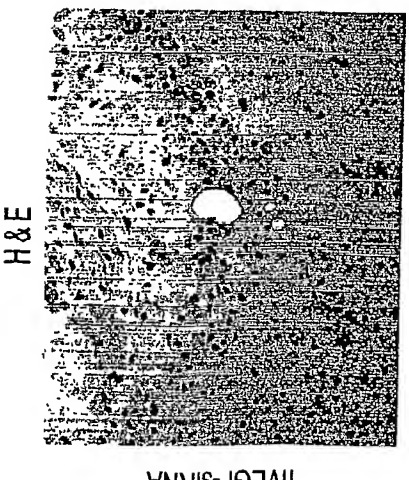


FIG. 8B

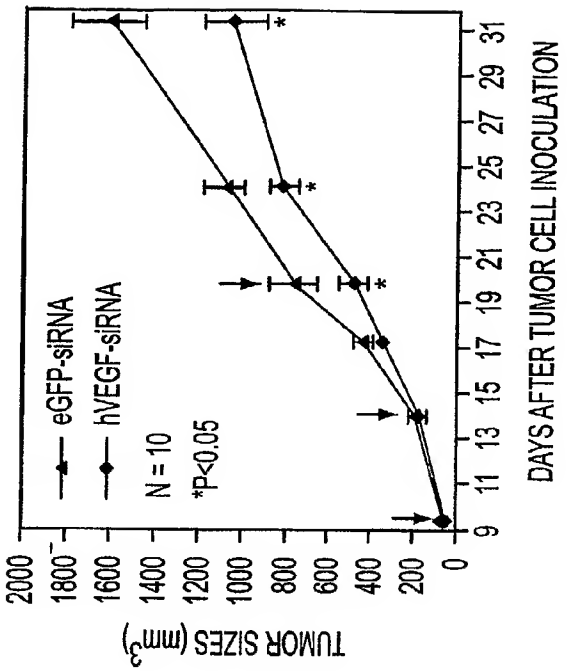

TUMOR SIZES (mm³)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
eGFP-siRNA
hVEGF-siRNA
N = 10
*P<0.05
*
9 11 13 15 17 19 21 23 25 27 29 31
DAYS AFTER TUMOR CELL INOCULATION

FIG. 9B

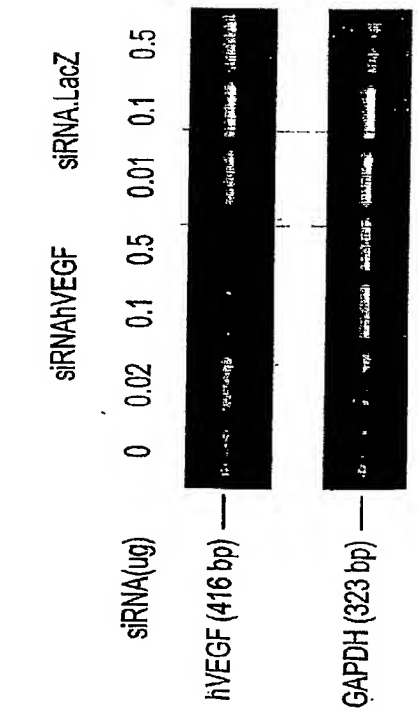

siRNA(ug)
0 0.02 0.1 0.5 0.01 0.1 0.5
siRNAhVEGF
siRNA.LacZ
hVEGF (416 bp)
GAPDH (323 bp)

FIG. 9A

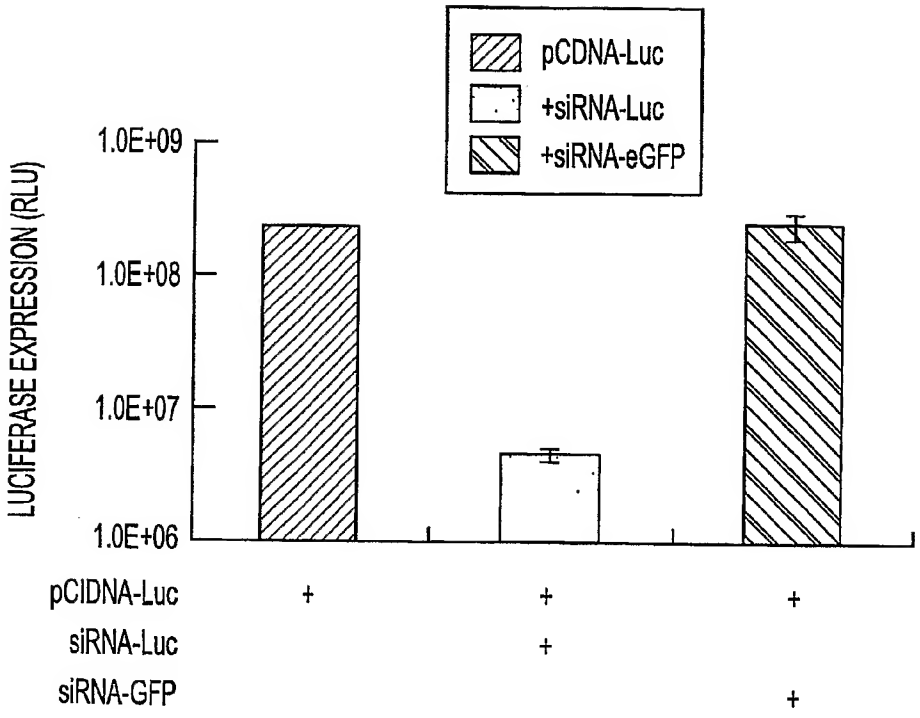

pCDNA-Luc
+siRNA-Luc
+siRNA-eGFP
LUCIFERASE EXPRESSION (RLU)
1.0E+09
1.0E+08
1.0E+07
1.0E+06
pCIDNA-Luc + + +
siRNA-Luc +
siRNA-GFP +

FIG. 10

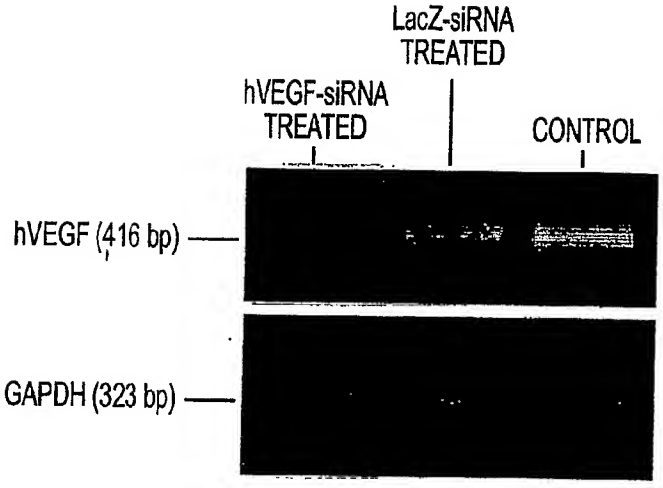

LacZ-siRNA
TREATED
hVEGF-siRNA
TREATED
CONTROL
hVEGF (416 bp)
GAPDH (323 bp)

FIG. 11

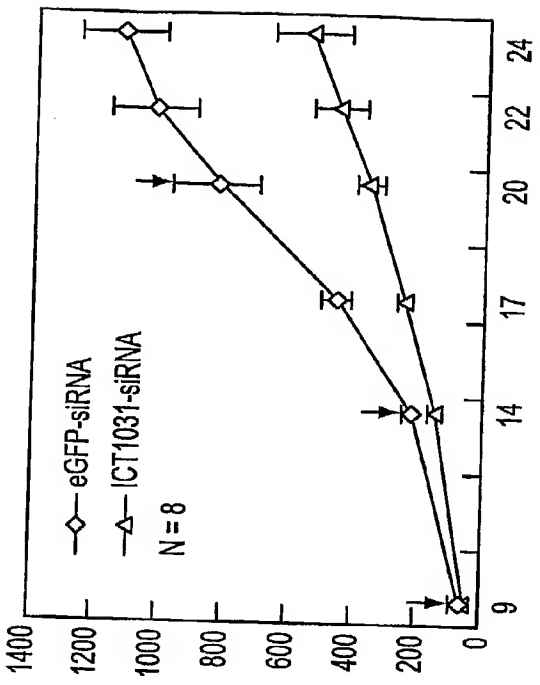


FIG. 12B

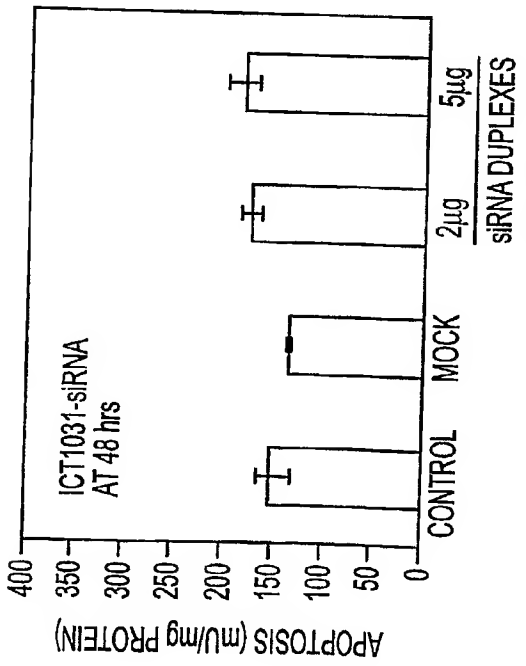


FIG. 12A

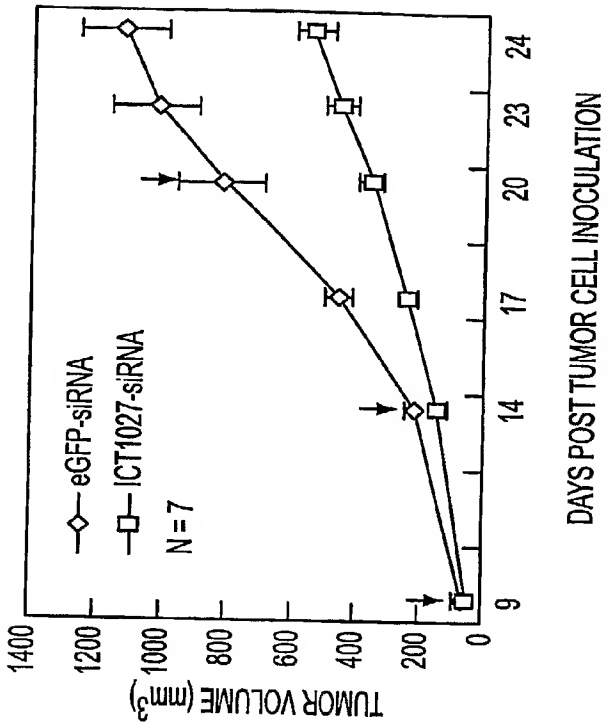


FIG. 13B

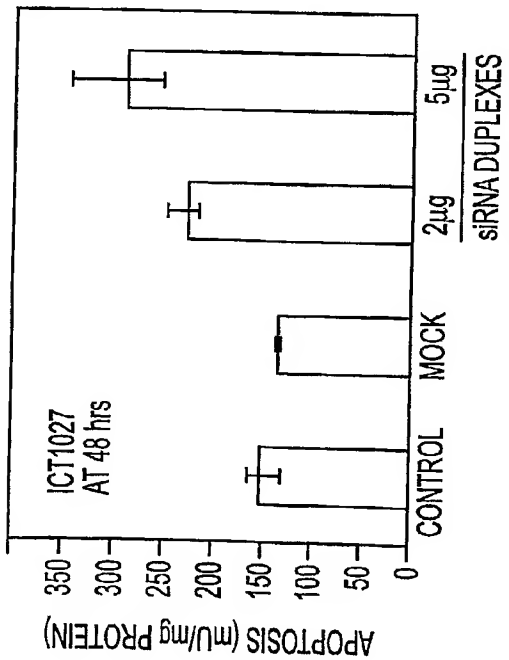


FIG. 13A

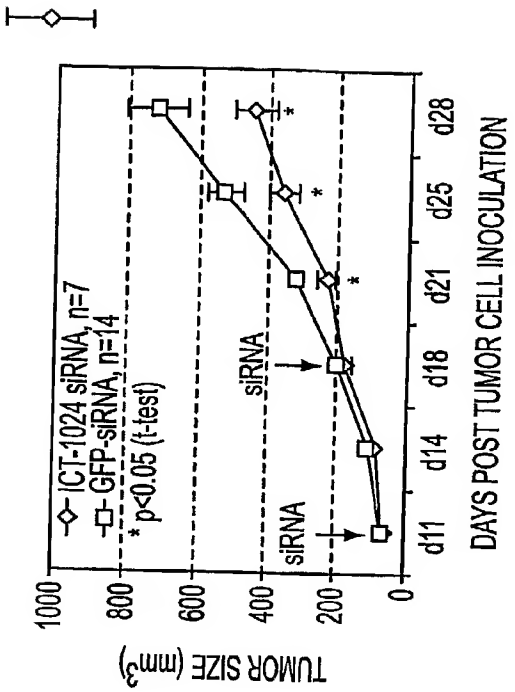


FIG. 14B

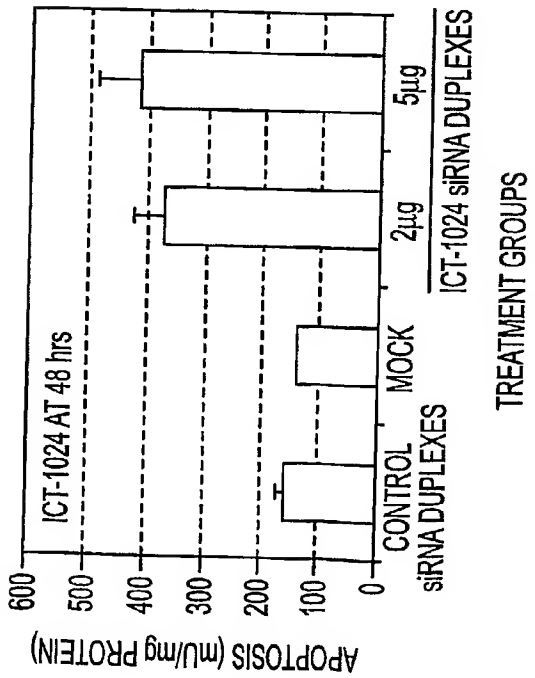


FIG. 14A

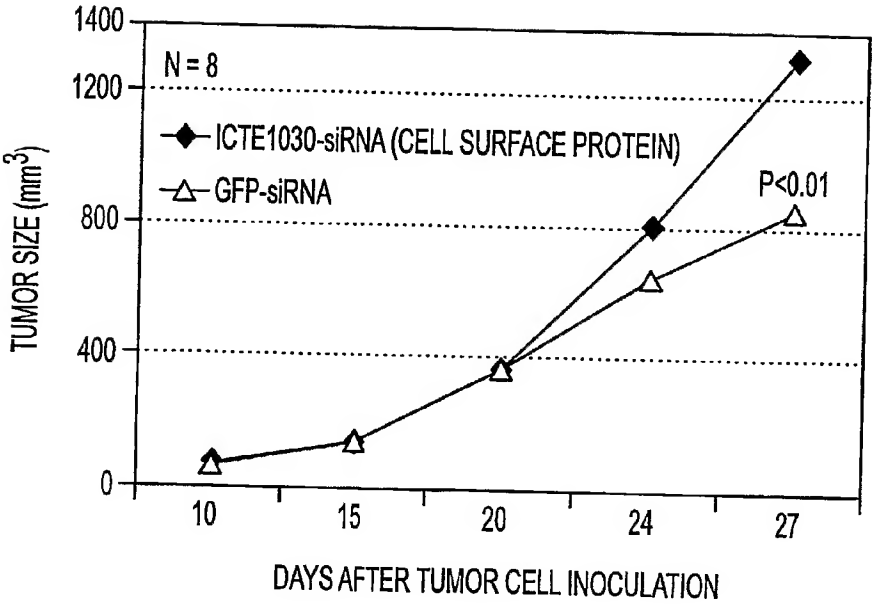

1400
1200
800
400
0
N = 8
ICTE1030-siRNA (CELL SURFACE PROTEIN)
GFP-siRNA
P<0.01
TUMOR SIZE (mm3)
10
15
20
24
27
DAYS AFTER TUMOR CELL INOCULATION

FIG. 15

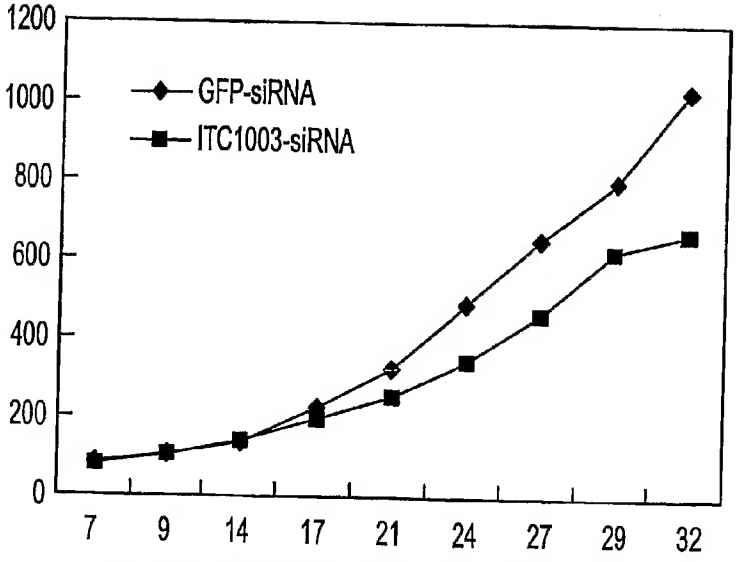

1200
1000
800
600
400
200
0
GFP-siRNA
ITC1003-siRNA
7
9
14
17
21
24
27
29
32
POLYTRAN-MEDIATED siRNA DELIVERY IN 435 TUMOR

FIG. 16

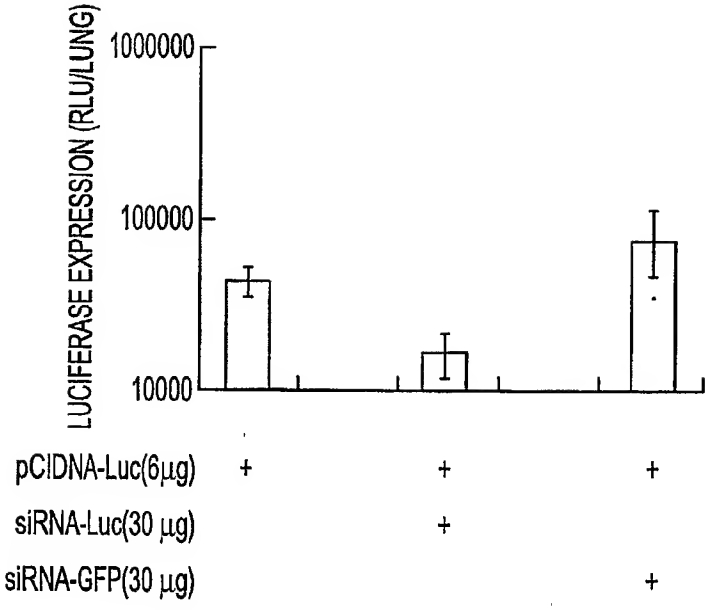

LUCIFERASE EXPRESSION (RLU/LUNG)
1000000
100000
10000
pCIDNA-Luc(6μg) + + +
siRNA-Luc(30 μg) +
siRNA-GFP(30 μg) +

FIG. 17

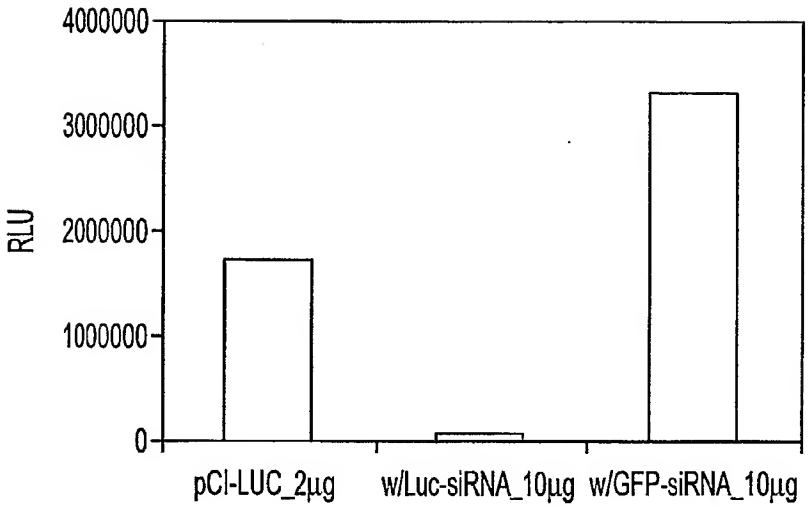

4000000
3000000
2000000
1000000
0
RLU
pCI-LUC_2µg
w/Luc-siRNA_10µg
w/GFP-siRNA_10µg

FIG. 18

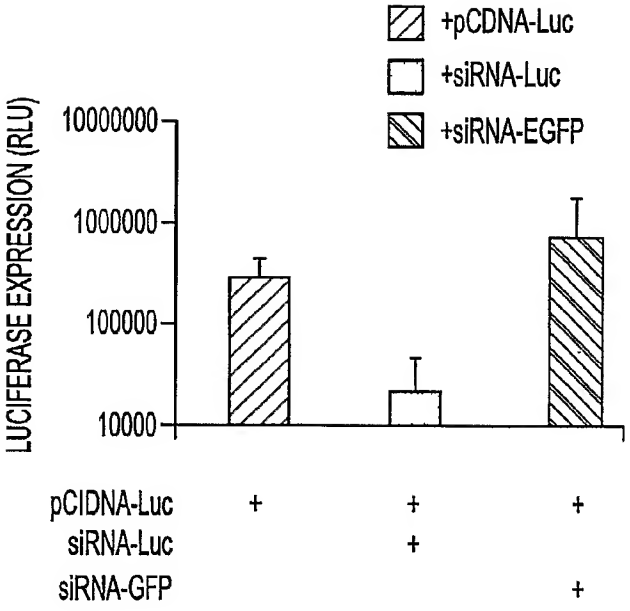

+pCDNA-Luc
+siRNA-Luc
+siRNA-EGFP
LUCIFERASE EXPRESSION (RLU)
10000000
1000000
100000
10000
pCIDNA-Luc + + +
siRNA-Luc +
siRNA-GFP +

FIG. 19

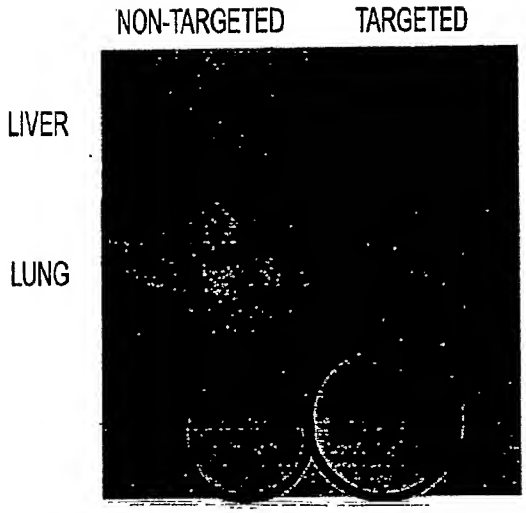


FIG. 20

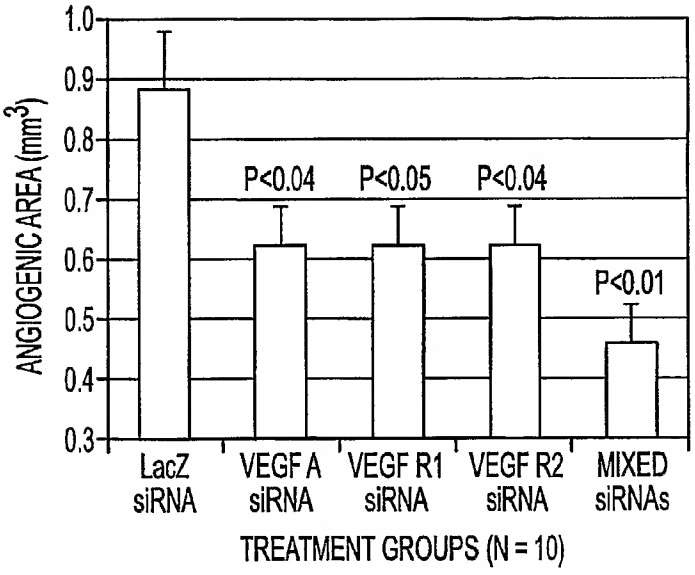
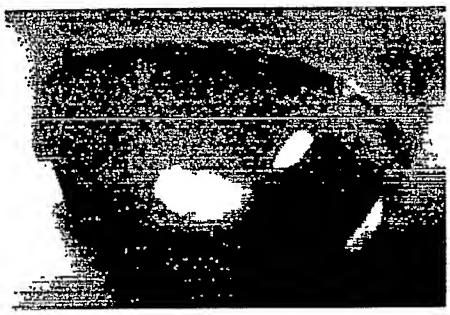
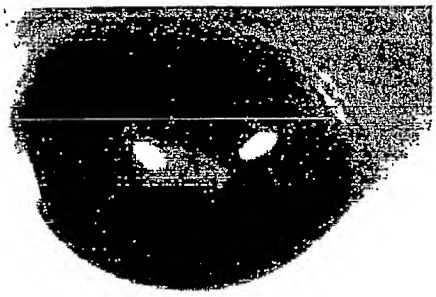

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
ANGIOGENIC AREA (mm3)
P<0.04
P<0.05
P<0.04
P<0.01
LacZ siRNA
VEGF A siRNA
VEGF R1 siRNA
VEGF R2 siRNA
MIXED siRNAs
TREATMENT GROUPS (N = 10)

FIG. 21



LacZ-siRNA

FIG. 22A



VEGFR1-siRNA

FIG. 22B

VEGF-siRNA

FIG. 22C

VEGFR2-siRNA

FIG. 22D

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: **INTRADIGM CORPORATION** [US/US]; 12115 Parklawn Drive, Suite K, Rockville, MD 20852 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SCARIA, Puthupparampil, V.** [US/US]; 9602 Swallow Point Way, Montgomery Village, MD 20886 (US). **WOODLE, Martin, C.** [US/US]; 8205 Beech Tree Road, Bethesda, MD 20817 (US). **LU, Patrick, Y.** [US/US]; 17093 Briardale Road, Rockville, MD 20855 (US). **TANG, Qingquan** [US/US]; 31 Longmeadow Drive, Gaithersburg, MD 20878 (US). **XU, Jun** [CN/US]; 18120 Coachmans Road, Germantown, MD 20874 (US). **XIE, Frank, Y.** [CN/US]; 13921 Rockingham Road, Germantown, MD 20874 (US).

(74) Agents: **BOOTH, PAUL M** et al.; HELLER EHRMAN WHITE & MCAULIFFE LLP, 1666 K Street, N.W., Suite 300, Washington, DC 20006-1228 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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26 August 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/013310 A3

(54) Title: METHODS OF DOWN REGULATING TARGET GENE EXPRESSION IN VIVO BY INTRODUCTION OF INTERFERING RNA

(57) Abstract: Methods and compositions are provided for down regulation of target gene expression in vivo by RNA interference. The methods are useful for target discovery and validation of gene-based drug development, and for treatment of human diseases.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/24587

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 21/02, 21/04; C12N 15/85, 15/86; C12P 19/34; C12Q 1/68
US CL : 435/6, 91.1, 325, 375; 514/44; 536/24.5, 24.3, 24.33, 24.31

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 91.1, 325, 375; 514/44; 536/24.5, 24.3, 24.33, 24.31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN, MEDLINE, CAPLUS, LIFESCI, EMBASE, USPATFULL, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99/53050 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 21 October 1999 (21.10.99), see entire document.	1-6, 21-22, 24-42, 44-45
A,P	CHECK, E. RNA to the rescue? Nature. 04 September 2003, Vol. 245, pages 10-12, see entire document.	1-6, 21-22, 24-42, 44-45
A,E	NOVINA et al. The RNAi revolution. Nature. 08 July 2004, Vol. 430, pages 161-164, see entire document.	1-6, 21-22, 24-42, 44-45
Y, P	US 6,657,054 B1 (SUN et al.) 02 December 2003 (02.12.03), see entire document.	1-6, 21-22, 24-42, 44-45
Y,P	US 6,506,559 B1 (FIRE et al.) 14 January 2003 (14.01.03), see entire document.	1-6, 21-22, 24-42, 44-45

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2004 (12.07.2004)	20 JUL 2004
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Karen A. Lacourciere Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/24587

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 7-20, 23 and 43
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.